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Antioxidant protection, carotenoids and the costs of immune challenge in greenfinches

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Summary

Costs accompanying immune challenges are believed to play an important role in life-history trade-offs and warranting the honesty of signal traits. We performed an experiment in captive greenfinches (*Carduelis chloris* L.) in order to test whether and how humoral immune challenge with non-pathogenic antigen [sheep red blood cells (SRBC)] affects parameters of individual condition including intensity of coccidian infection, estimates of total antioxidant protection, plasma carotenoids and ability to mount a cell-mediated immune response. We also asked whether the potential costs of immune challenge can be alleviated by dietary carotenoid supplementation. None of the treatments affected intensity of coccidiosis. Humoral immune challenge suppressed the cell-mediated response to phytohemagglutinin (PHA), suggesting a trade-off between the uses of different arms of the immune system. Immune challenge reduced body-mass gain, but only among the carotenoid-depleted birds, indicating that certain somatic costs associated with immune system activation can be alleviated by carotenoids. No evidence for oxidative stress-induced immunopathological damages could be found because immune activation did not affect total antioxidant protection or carotenoid levels. Carotenoid supplementation inclined birds to fattening, indicating that lutein interfered with lipid metabolism. Altogether, our results support the hypotheses of biological importance of carotenoids and exemplify the overwhelming complexity of their integrated ecophysiological functions.

Key words: immune challenge, phytohemagglutinin, plasma carotenoids, SRBC, total antioxidant capacity.

Introduction

Actual and potential damage caused by parasites and pathogens has led host species to develop sophisticated protection mechanisms, ranging from behavioural defences and physical barriers to the immune system. Using the immune system can be costly, as revealed by studies demonstrating the adverse effects of activation of some components of immune defence on reproductive effort (e.g. Ilmonen et al., 2000), ornamental trait expression (e.g. Kilpimaa et al., 2004), cognitive performance (e.g. Mallon et al., 2003), other components of immune defence (Kidd, 2003) and even survival (Moret and Schmid-Hempel, 2000; Victor and de la Fuente, 2003; Hanssen et al., 2004). However, the question as to what exactly makes the activation of immune defences costly has remained less clearly understood (e.g. Schmid-Hempel, 2002; Zuk and Stoehr, 2002). The traditional view of ecologists, namely that the costs involved in life-history trade-offs are basically energetic, has gained equivocal support in immunoecological studies (reviewed by Råberg et al., 2002; Demas, 2004; Eraud et al., 2005); an alternative hypothesis is that costs of immune responses are primarily caused by the accompanying immunopathological damages (Råberg et al., 1998; Westneat and Birkhead, 1998).

An important source of immunopathology is oxidative stress, caused by excess production of reactive compounds during immune responses (Halliwell and Gutteridge, 1999). Oxidative products and free radicals, which are highly reactive by-products of normal metabolism and immune defences, can cause extensive damage to nucleic acids, proteins and lipids if an organism lacks sufficient antioxidant protection (Halliwell and Gutteridge, 1999). To control and neutralise free radicals, animals maintain a system of defences based on different antioxidants. Endogenous antioxidants (like uric acid, bilirubin and albumin, and enzymes such as catalase, superoxide dismutase and glutathione peroxidase) are synthesized by an organism whereas exogenous antioxidants (like vitamins E and A, and carotenoids) must be obtained from food.

Of all the antioxidants, animal ecologists have paid a disproportionate amount of attention to carotenoids (Lozano, 1994; Olson and Owens, 1998; von Schantz et al., 1999; Møller et al., 2000; McGraw, 2006). Carotenoid-based visual characters enable individuals to signal their phenotypic and/or

genetic quality: if an individual has only a limited amount of carotenoids at its disposal, then it can use them for signals only when it does not need them for maintenance (Lozano, 1994). Hence, carotenoid-based traits might either signal foraging (and food absorption) efficiency, immunocompetence or anti-oxidative potential of signallers. The relative importance of these factors is currently under lively debate (Hill, 1999; Lozano, 2001; Hartley and Kennedy, 2004).

Compared with their role in signalling and immunity, the antioxidant function of carotenoids has remained much more poorly understood, even in traditional mammal models (El-Agamey et al., 2004). The situation is even more complicated with birds. Given that most avian species live longer than similar-sized mammals despite their higher metabolic rates, birds are thought to have evolved unique protective mechanisms against oxidative damage (Klandorf et al., 2001). With few exceptions (e.g. Woodall et al., 1996; Jaensch et al., 2001), antioxidant properties of carotenoids in birds have been predominantly studied in the context of embryo-protective maternal effects (Surai, 2002; McGraw et al., 2005a), and only few studies (Alonso-Alvarez et al., 2004; Bertrand et al., 2006; Costantini et al., 2006) have measured the relationships between carotenoids and general antioxidant defences in nestlings or adults.

Here we address the questions about the role of carotenoids in modulation of oxidative stress *via* changes of total antioxidative potential and physiological consequences of immune challenges in captive greenfinches. (1) Under the hypothesis that activation of the immune system by a novel antigen weakens antioxidant protection, we predicted that an immune challenge results in a reduction of plasma carotenoid levels and total antioxidant protection. If mounting an immune response impairs the general physiological condition of an individual, we also expected to find an effect of immune challenge on the indices of individual nutritional state. (2) If a trade-off exists between different arms of the immune system, so that eliciting a humoral response diverts resources away from the cell-mediated response, we predicted that birds injected with sheep red blood cells (SRBC) will produce a weaker cutaneous swelling in response to mitogen [phytohemagglutinin (PHA)] injection. This prediction is based on the allocation principle, which underlies the rationale of ecological immunology (e.g. Sheldon and Verhulst, 1996), and on the evidence regarding cross-regulation between the different components of the immune system from mammal models (e.g. Kidd, 2003). (3) Under the hypothesis that carotenoids are involved in antioxidant protection and general health maintenance, we predicted that these potential costs of immune challenge (i.e. reduced antioxidant protection and nutritional state) will be alleviated among the birds receiving dietary carotenoid supplementation. (4) We also predicted that carotenoid-supplemented birds mount a stronger immune response against foreign antigens than control individuals if carotenoids exert an immunostimulatory effect in our model system. (5) Finally, assuming that carotenoids significantly contribute to total antioxidativity, we predicted that individual plasma-carotenoid levels correlate positively with measures of total antioxidant capacity.

Materials and methods

Greenfinches weigh approximately 30 g and are sexually dichromatic, gregarious granivorous passerines native to the western Palearctic region. Carotenoid-based plumage coloration in males is sexually selected (Eley, 1991) and negatively affected by intestinal (Hõrak et al., 2004), viral (Lindström and Lundström, 2000) and hematozoan (Merilä et al., 1999) infections. Fifty-six male greenfinches were caught in mist-nets at the Sõrve Bird Observatory (Saaremaa, Estonia; 57°55′N; 22°03′E) on 25 (day 0) and 26 January 2005. At capture, 13 of these individuals were blood sampled during the first morning hours to obtain natural background levels for the concentration of carotenoids. Birds were transported to Tartu and housed in individual indoor cages (27×51×55 cm) with sand bedding. Mean temperature in the aviary during the experiment was 14.6±1.2°C (s.d.) and mean humidity was 55.5±7.6% (s.d.). The birds were supplied *ad libitum* with sunflower seeds and filtered tap water. Birds were held on the natural day-length cycle. All blood samples were collected before the lights were turned on, in order to obtain the values of biochemical parameters characteristic to the state of overnight fast. Birds were released to their natural habitat on 19 March 2005 (day 53). The study was conducted under license from the Estonian Ministry of the Environment.

Experimental procedure

The experimental procedure is described in Fig. 1. After transportation to Tartu, birds were allowed a 13-day acclimatisation period (days 2–15). Birds were divided into four equal (14 birds) treatment groups that were set to have similar average body mass at capture and age composition (five first-year and nine older birds in each group). On the morning of day 15, pre-experimental blood samples were collected and the birds were assigned to 2×2 treatments by immune challenge and carotenoid supplementation. The immunochallenged group (28 birds) received an injection of 50 μl of 40% suspension of SRBC in isotonic saline into the pectoralis muscle on days 15 and 31 (Fig. 1). Controls were injected with the same amount of isotonic saline only. Half the birds started to receive carotenoid supplementation in their diet on the same day. Supplementation consisted of a 10 $\mu g\ ml^{-1}$ water solution of lutein and zeaxanthin (20:1, w/w), prepared from OroGlo liquid solution of 11 $g\ kg^{-1}$ xanthophyll activity (Kemin AgriFoods Europe, Herentals, Belgium). These solutions were freshly prepared each morning using filtered (Brita® Classic; BRITA GmbH, Taunusstein, Germany) tap water at 4°C and were provided in 30-ml doses in opaque dispensers in order to avoid oxidation of carotenoids. Control birds received filtered tap water.

On day 21 (seven days after treatments started), a second blood sample was collected (‘First titre’ in Fig. 2). On the next day, all the birds were injected intradermally in the wing web

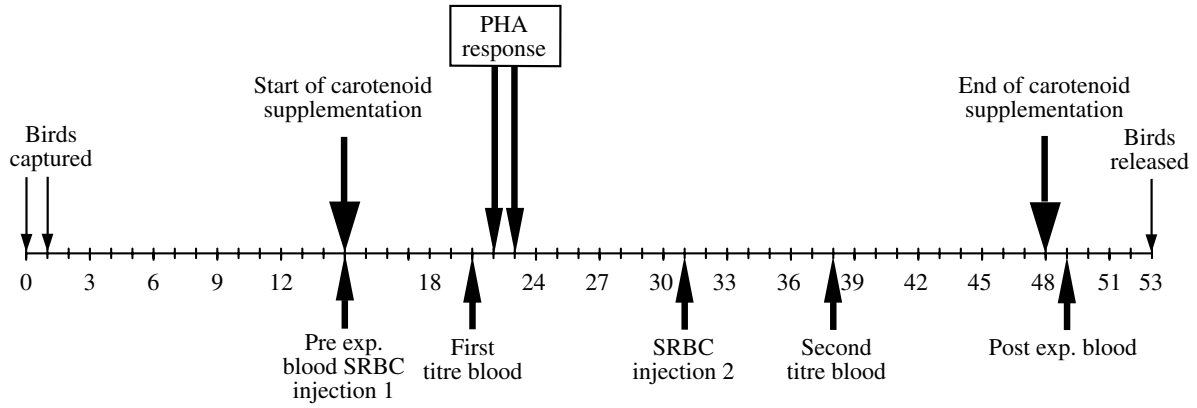

Fig. 1. Experimental process. Day 0=25 January 2005.

with 0.2 mg of PHA in 0.04 ml of isotonic saline. The swelling response was measured 24 h later. On day 31 (10 days after primary immunisation), previously immunised birds were again injected with the same dose of SRBC and control birds received a second saline injection. Blood for secondary-antibody titres ('Second titre' in Fig. 2) was collected eight days after secondary immunisation (Fig. 1). Carotenoid supplementation was ended on the 48th day of the experiment and the last ('Post exp.') blood sample was collected the following day (Fig. 1). In all cases, 100–300 μl of blood was drawn from brachial or tarsal veins and, after centrifugation, serum (for antibody measurement) and plasma (for the remaining analyses) were stored at –75°C until analysed. All spectrophotometric analyses were performed with a Tecan microplate reader (Model Sunrise; Tecan Austria GmbH, Grödig/Salzburg, Austria). During the few hours between

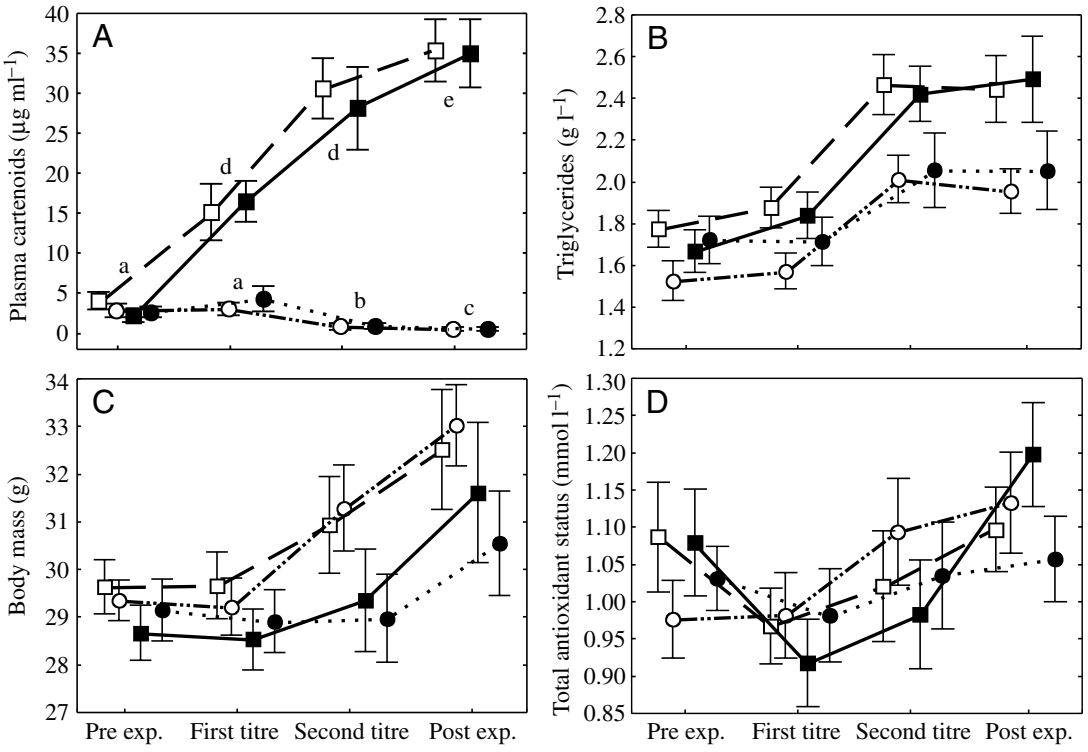

Fig. 2. Effects of carotenoid supplementation and immune challenge on physiological parameters of greenfinches. For carotenoids, individual changes are tested with Wilcoxon matched-pairs tests and between-treatment differences with Mann–Whitney *U*-tests. Carotenoid concentrations, marked with different letters, are significantly different from each other ($P<0.05$). Group sizes range from five to eight on the first-titre measurement and from 11 to 14 on other occasions. Mean-trait values, exact sample sizes and statistics are presented in Table 1. For other variables, *P*-values for time × treatment interaction terms are presented in the text, and in most cases each group contains 14 individuals. Open squares, carotenoid–saline; filled squares, carotenoid–SRBC; filled circles, water–SRBC; open circles, water–saline.

blood collection and centrifugation, samples were maintained in a refrigerator at 4°C.

In the course of study, birds were monitored for their individual levels of coccidian infection by fecal examination. The coccidian species present in the feces of migrating greenfinches in Estonia has been previously identified as *Isospora lacazei* (for details, see Hõrak et al., 2004). Coccidian-infection intensities (number of oocysts per gram of feces) of individual greenfinches were quantified as described previously (Hõrak et al., 2004; Hõrak et al., 2006; Saks et al., 2006). Infection intensities were determined on days 4, 6, 8, 10, 12, 16, 18, 20–22, 24–34, 36, 37, 39, 41, 44, 47 and 48 of the experiment. All the birds carried infections when brought into the aviary. On days 24–30, all the birds received sulphonamide-coccidiostatic treatment (sulfathiazole sodium pentahydrate, 2 g l^{-1} water) in their drinking water in order to equalise their infection status. The treatment, however, was not fully efficient because by the end of treatment (day 25), 27% (15/56) of the birds were still shedding oocysts. The effect of treatment almost completely vanished by day 34, when 95% of birds (53/56) had started to shed oocysts again.

Assessment of immune function

Immune response against SRBC involves both B- and T-lymphocytes and is used for routine evaluation of humoral immunity in immunological, immunotoxicological and ecological studies (see Hõrak et al., 2003). Anti-SRBC antibody titres were measured using a hemagglutination assay (Wegmann and Smithies, 1966; Lawler and Redig, 1984) as described in detail by Saks et al. (Saks et al., 2003), with the exception that 25 μl of serum and 25 μl of isotonic saline were pipetted into the first well of a microtitre plate. This mixture was serially diluted using 25 μl of saline. Hence, we used initial plasma concentrations that were four times higher than in our previous studies (Hõrak et al., 2003; Saks et al., 2003), which enabled us to detect antibody concentrations that were four times lower than previously. (Yet no antibodies were detected in the serum of non-immunized birds.) Titre was scored as the number of wells in a dilution row that contained a sufficient amount of antibodies to hemagglutinate SRBC.

Cutaneous hypersensitivity reaction, resulting from PHA injection, reflects the combined responses of T-cells, cytokines and inflammatory cells (e.g. Stadecker et al., 1977). This assay has become increasingly popular in avian studies, where it is considered as a proxy of cell-mediated immune responsiveness (see Smits et al., 1999). We followed the simplified protocol (Smits et al., 1999) as described in detail by Saks et al. (Saks et al., 2003). The repeatability (Lessells and Boag, 1987) of swelling response, based on three consecutive measurements, was 0.88 (F=22.9; P<0.0001; N=49).

Indices of nutritional state

To assess nutritional state, we measured body mass (before blood samplings) and plasma triglyceride concentrations. High blood triglyceride levels are indicative of a resorptive state during which lipid is formed by the liver and deposited in muscle and adipose tissues. Hence, triglyceride concentrations reflect the individual's state of fattening by indicating the amount of lipids absorbed during the few hours before blood sampling (Jenni-Eiermann and Jenni, 1998). Blood was collected after nocturnal fast, so plasma triglyceride levels in our study reflect the variation in basic nutritional state, independently of recent food intake. Concentrations were determined by enzymatic colorimetric test (GPO-PAP method) (Human GmbH, Wiesbaden, Germany).

Carotenoids

The most prevalent carotenoids in the plasma of greenfinches are lutein and its structural isomer, zeaxanthin (McGraw, 2004). Concentrations of carotenoids were determined spectrophotometrically (e.g. Tella et al., 1998; Bortolotti et al., 2000; Peters et al., 2004) using acetone-resistant microtitre plates. Acetone (150 μl) was added to 15 μl of plasma and centrifuged for 10 min at 16 800 ***g***. Absorbance of supernatant was measured at 449 nm, corresponding to the maximum absorbance of lutein in acetone (Zsila et al., 2005). Calibration curves were prepared using lutein (X-6250; Sigma, St Louis, MO, USA) as standard. Repeatability (Lessells and Boag, 1987) of carotenoid measurements between different microtitre plates was 0.95 ($F_{15,20}$=46.0; P<0.0001).

Total antioxidativity

Two methods, based on the capacity of biological fluids to inhibit redox reaction induced by free radicals, were used for assessment of total antioxidant capacity of plasma. A total antioxidant status (TAS) assay was performed, adapting the commercially available kit (Randox Laboratories, Crumlin, UK) for small (5 μl) plasma samples. This assay (sometimes also termed TEAC) is widely used in clinical studies (Dotan et al., 2004). In this assay, azino-diethyl-benzthiazoline sulphate (ABTS) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$. This has a relatively stable blue-green colour, which is measured at 600 nm. Antioxidants in the plasma cause suppression of this colour production to a degree that is proportional to their concentration. TAS is expressed in mmol l^{-1}. Repeatability (Lessells and Boag, 1987) of TAS values among individual samples, measured on different plates, was 0.93 ($F_{14,15}$=29.2; P<0.0001).

Total antioxidant potential (AOP) was estimated using the BIOXYTECH® AOP-490™ assay (OxisResearch™, Portland, OR, USA), which is based upon the reduction of Cu^{2+} to Cu^+ by the combined action of all antioxidants presented in a sample. A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a 2:1 complex with Cu^+, which has a maximum absorbance at 490 nm. Colour change of the plasma, incubated with reagent containing Cu^{2+} and chromogen for 3 min at room temperature, is measured. A standard of known uric acid (a water-soluble antioxidant) concentration was used to create the calibration curve, so the results are quantified in mmol l^{-1} uric acid equivalents. The assay was adapted for small (5 μl) plasma samples.

Repeatability of AOP values among individual samples, measured on different plates, was 0.88 ($F_{14,14}$=15.2; P<0.0001).

Statistics

Effects of experimental treatments on the dynamics of body mass, triglycerides and TAS were analysed by repeated-measures analysis of variance (ANOVA), dropping non-significant interaction terms from the final models. Assumptions for the parametric analyses were met for these variables. Since carotenoid concentrations were not normally distributed among unsupplemented birds, we could not apply repeated-measures ANOVA for testing the treatment effects. Therefore, individual changes within treatment groups were tested with Wilcoxon matched-pairs tests and between-treatment differences with Mann–Whitney *U*-tests (Table 1). Age (first-year *versus* older) did not affect any of the studied parameters. Non-parametric tests were applied for analyses of coccidian-infection intensities because these were not normally distributed. *P*-values are for two-tailed tests. Sample sizes differ between some analyses because of our inability to collect a sufficient amount of blood from all the birds. Mean trait values are presented with ± s.d.

Results

Physiological effects of carotenoid supplementation and immune challenge

Plasma carotenoid concentration at capture ranged from 1.5–21.6 μg ml^{-1}, averaging at 10.2±6.6 μg ml^{-1} (N=13). After 15 days spent in captivity, average carotenoid levels in greenfinches had decreased more than twofold (to 3.8±2.7 μg ml^{-1}) as compared with the levels at capture (Z=3.1, P=0.002, Wilcoxon matched-pairs test). During the experiment, plasma carotenoid levels in unsupplemented birds continued to decline, whereas among the supplemented birds, average plasma-carotenoid concentrations steadily increased (Fig. 2A). Immunisation with SRBC had no effect on the dynamics of plasma carotenoid levels in either group (P=0.5–1; see Table 1 for details).

Carotenoid supplementation increased plasma-triglyceride levels in the second half of the experiment; however, triglycerides were not affected by the immune challenge [Fig. 2B; $F_{3,141}$=3.33, P=0.021 for time × carotenoid interaction term and $F_{3,141}$=0.11, P=0.952 for time × SRBC interaction term in repeated-measures ANOVA with main effects of carotenoid ($F_{1,47}$=8.46, P=0.005) and SRBC ($F_{1,47}$=0.194, P=0.662) treatments and time ($F_{3,141}$=44.17, P<0.0001)].

Body mass of birds increased during the second half of the experiment (Fig. 2C). Mass dynamics in the whole sample was not affected by carotenoid supplementation or immunisation treatments [$F_{3,141}$=1.53, P=0.210 for time × carotenoid interaction term and $F_{3,141}$=0.35, P=0.786 for time × SRBC interaction term in repeated-measures ANOVA with main effects of carotenoid ($F_{1,47}$=0.03, P=0.865) and SRBC ($F_{1,47}$=0.05, P=0.824) treatments and time ($F_{3,141}$=5.66, P=0.001)]. However, the effect of immunisation on body-mass dynamics emerged when unsupplemented birds were analysed separately ($F_{3,78}$=10.7, P=0.008 for time × SRBC interaction term in repeated-measures ANOVA with main effects of SRBC treatment ($F_{1,26}$=1.9, P=0.193) and time ($F_{3,78}$=43.3, P<0.00001)]. This was because immunised birds gained body mass more slowly than non-immunised birds (Fig. 2C).

None of our treatments affected plasma TAS [Fig. 2D; $F_{3,141}$=1.52, P=0.209 for time × carotenoid interaction term and $F_{3,141}$=0.35, P=0.785 for time × SRBC interaction term in repeated-measures ANOVA with main effects of carotenoid and SRBC treatments ($F_{1,46}$=0.03–0.05, P=0.8) and time ($F_{3,141}$=5.67, P=0.001)]. Similarly, plasma AOP did not differ between treatments after secondary immunisation ($F_{3,52}$=0.47, P=0.701) or at the end of experiment ($F_{3,37}$=0.47, P=0.705).

Swelling response to PHA injection tended to be lower among SRBC-injected birds (0.34±0.18 mm, N=26) than among unsupplemented birds (0.45±0.23 mm, N=23; t=1.90, P=0.064). This effect of treatment became significant ($F_{1,45}$=4.76, P=0.034) after inclusion of body mass at capture [$F_{1,45}$=7.77, P=0.008, β=0.37±0.13 (s.e.m.)] as a covariate into an analysis of covariance (ANCOVA) model.

Table 1. *Mean plasma carotenoid concentrations (μg ml^{-1}) in different treatment groups and P-values and Z-statistics for between-group comparisons (Mann–Whitney U-tests)*

	Control			Carotenoid			Difference between control and carotenoid
	SRBC	Saline		SRBC	Saline		
Date	Mean ± s.d. (N)	Mean ± s.d. (N)	P diff. (Z)	Mean ± s.d. (N)	Mean ± s.d. (N)	P diff. (Z)	P diff. (Z)
9 Feb	2.6±2.3 (13)	2.8±3.2 (14)	0.846 (0.19)	2.2±2.9 (14)	4.1±4.2 (14)	0.206 (1.26)	0.859 (0.17)
15 Feb	4.3±3.8 (6)	3.1±.3 (8)	0.796 (0.25)	16.5±6.3 (6)	15.1±7.9 (5)	0.715 (0.36)	0.00005 (4.05)
4 March	0.9±1.1 (12)	0.8±1.5 (12)	0.644 (0.46)	28.2±16.3 (10)	30.6±13.6 (13)	0.660 (0.43)	<0.00001 (5.51)
15 March	0.5±0.7 (12)	0.5±0.9 (14)	0.554 (0.59)	35.0±14.7 (13)	35.4±12.8 (11)	0.950 (0.06)	<0.00001 (5.98)

Individual changes in carotenoid concentrations among control birds: 9 Feb–15 Feb: P=0.362; Z=0.91; N=14. 15 Feb–4 March: P=0.006; Z=2.75; N=11. 4 March–15 March: P=0.004; Z=2.84; N=23. Individual changes in carotenoid concentrations among carotenoid-supplemented birds: 9 Feb–15 Feb: P=0.003; Z=2.93; N=11. 15 Feb–4 March: P=0.138; Z=1.48; N=9. 4 March–15 March: P=0.006; Z=2.73; N=19. Wilcoxon matched-pairs tests.

Pre-experimental coccidian-infection intensity (averaged over first five days when infection was measured) did not differ between the experimental groups (H=0.37, N=56, P=0.946; Kruskal–Wallis ANOVA). Infection intensities at the end of the experiment (averaged over days 47–48) were not affected by the carotenoid treatment (Z=0.4, N=55; P=0.711; Mann–Whitney U-test) or immune challenge (Z=0.4, N=55; P=0.686; Mann–Whitney U-test).

Effects of carotenoid supplementation on the immune responses

Twenty-four out of 28 immunised birds produced a detectable amount of antibodies against SRBC after primary immunisation, and 26 out of 28 birds responded to secondary immunisation. We could not detect a difference in the magnitude of immune responses between carotenoid-supplemented and unsupplemented birds (Z=1.65 and 0.96, P=0.10 and 0.33, for primary and secondary titres, respectively). Furthermore, after the exclusion of nonresponsive individuals (i.e. the birds with 0-titres) from the sample, primary immune response was significantly higher in the unsupplemented group (6.76±1.21, N=12 *versus* 5.50±1.39, N=12; t=2.35, P=0.028).

Swelling response to PHA injection did not differ significantly between supplemented (0.42±0.23 mm, N=26) and unsupplemented birds (0.38±0.19 mm, N=23; t=0.77, P=0.445). Inclusion of the SRBC immunisation as a factor in the ANOVA model did not affect the significance of the carotenoid-treatment term. PHA response did not correlate with individual plasma-carotenoid levels (r_s=0.06–0.11; P=0.6).

Correlations between indices of total antioxidativity, carotenoids and infection

Our two measures of antioxidant protection, TAS and AOP, correlated positively in all three measuring occasions (r=0.52–0.81; P=0.015–<0.0001; N=12–56; Fig. 3). However, none of these measures correlated significantly with plasma carotenoid levels (r=–0.10–0.19; P=0.2–1.; N=25–55). High pre-experimental infection intensities were accompanied by low plasma-carotenoid levels (r_s=–0.36; N=55; P=0.007). None of the other health parameters correlated significantly with pre-experimental (r_s=–0.05–0.08; P=0.5–0.9; N=56) or post-experimental (r_s=–0.07–0.09; P=0.6–0.9; N=41–55) infection intensities.

Discussion

Dietary manipulation of carotenoid availability in our experiment was effective, resulting in almost total depletion of plasma carotenoid levels in the unsupplemented group. After 33 days of dietary supplementation, mean carotenoid concentrations of supplemented birds exceeded those of unsupplemented birds by approximately 70-fold. By the end of the experiment, plasma carotenoid levels among the supplemented birds were more than three times higher (35 μg ml^{-1}) than carotenoid levels at capture (10 μg ml^{-1}).

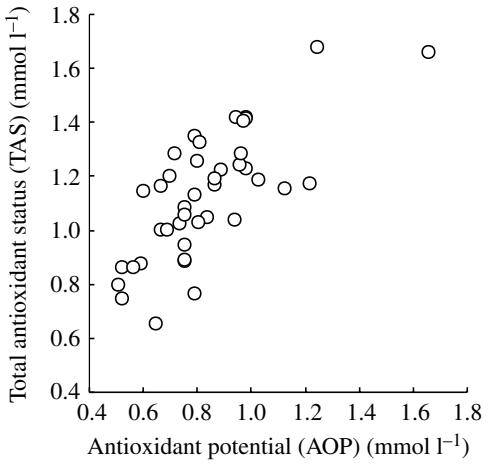

Fig. 3. Relationship between two measures of plasma antioxidant activity (TAS and AOP) at the end of the experiment (r=0.75; P<0.00001; N=41).

However, we are confident that such concentrations remain within the physiological range because the chroma of tail feathers, plucked and regrown during the experiment, remained approximately 10% lower than chroma of naturally grown feathers (U.K., L.S. and P.H., unpublished data). This indicates that plasma carotenoid levels of free-living greenfinches during moult exceed those obtained in this study. Relatively low carotenoid levels at capture in our study can probably be explained by the diet at capture site, where the birds predominantly fed on supplemented sunflower seeds, which have extremely low carotenoid content (McGraw et al., 2001).

Costs of immune response

Assuming that use of the immune system is costly, we predicted that mounting an immune response against SRBC would result in reduced antioxidant protection and plasma carotenoid levels, as well as impaired physiological condition and cell-mediated immunoresponsiveness. Of these parameters, the immune activation most clearly affected the swelling response to PHA. Adjusting for between-individual variation in physiological condition (estimated on the basis of body mass at capture), birds immunized with SRBC produced weaker cutaneous swelling response to PHA than sham-immunised birds on the seventh day after SRBC injection. This result points to the possible trade-off between the use of the different arms of the immune system, which is compatible with the general logic of ecological immunology (e.g. Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). This trade-off might be rooted in the basis of cross-regulation between humoral and cell-mediated immune responses (Mosmann and Coffman, 1989) in which (humoral) Th2 responses exert anti-inflammatory action by negatively regulating Th1-cell-mediated immunity (and *vice versa*). Although such cross-regulations have been frequently observed in mammal models (reviewed by Kidd, 2003), the discovery of a suppressed cutaneous swelling response in response to humoral immune

system activation in greenfinches is, to our knowledge, the first such evidence in birds. The generality of this phenomenon, however, is unclear as no suppression of PHA response by SRBC injection was observed in nestling western bluebirds (*Sialis mexicana*) (Fair and Myers, 2002) or growing quail (*Coturnix coturnix japonica*) (Fair et al., 1999).

Another piece of evidence about the possible costs of humoral immune activation originates from the data about body-mass dynamics. SRBC challenges significantly slowed down the increase of body mass during the second half of the experiment, but only for birds in the carotenoid-free diet (Fig. 2C). The findings of reduced body mass, mass gain or growth in response to non-pathological immune challenge have been documented in several avian studies (e.g. Klasing et al., 1987; Fair et al., 1999; Ots et al., 2001; Bonneaud et al., 2003) (but see Whitaker and Fair, 2002; Hõrak et al., 2000; Hõrak et al., 2003). Possible mechanisms include energy reallocation from maintenance to immune function (reviewed by Lochmiller and Deerenberg, 2000; Demas and Sakaria, 2005) or inflammation-induced sickness syndrome, which results in reduced food intake and locomotory activity (e.g. Bonneaud et al., 2003; Klasing, 2004). In this context, our result regarding the lack of effect of immune challenge on body-mass dynamics among carotenoid-supplemented birds is particularly interesting because it suggests that some physiological costs associated with immune system activation can be alleviated by the carotenoid supplementation. This effect was probably related to the enhancement of fat deposition among carotenoid-fed birds (Fig. 2B).

Assuming that possible immunopathological effects of SRBC challenge emerge because of excessive reactive-species production, we expected immunisation treatment to affect the biomarkers of antioxidant protection. However, despite the above-mentioned physiological effects of immune challenge, we did not detect any carotenoid depletion among SRBC-injected birds (Fig. 2A). This result is inconsistent with Alonso-Alvarez et al. (Alonso-Alvarez et al., 2004), who showed that immune challenge with a bacterial lipopolysaccharide (LPS), significantly depressed plasma carotenoid levels in captive zebra finches (*Taeniopygia guttata*). Similar results were obtained with chickens (*Gallus gallus domesticus*), where carotenoid depletion from the plasma and other tissues was specifically associated with markers of acute-phase response, such as interleukin-1 (Koutsos et al., 2003). In mallards (*Anas platyrhynchos*), higher anti-SRBC antibody titres were associated with a greater decline of plasma carotenoids (Peters et al., 2004). More generally, reduced levels of plasma lutein have also been associated with markers of inflammation in human studies (e.g. Kritchevsky et al., 2000; Gruber et al., 2004).

Depletion of plasma carotenoids during the inflammatory response might occur for several reasons. One possibility is that carotenoids might be incorporated into lymphoid tissues, where they act as immunomodulatory agents. In addition, carotenoids could be depleted from the plasma as antioxidants because of excess production of reactive species during the oxidative burst associated with inflammatory response (e.g. Walrand et al., 2005). However, it is also possible that changes of carotenoid metabolism during the acute-phase response are an indirect result of alterations in lipid metabolism without any carotenoid-specific regulation of tissue uptake (Koutsos et al., 2003). In any case, immune challenge with SRBC in captive greenfinches seemed to elicit much weaker inflammatory impact than LPS treatment in other species, which is perhaps not surprising given that SRBC, unlike LPS, might not stimulate robust innate immune responses (Klasing, 2004) (but see Eraud et al., 2005). This explanation would also be compatible with our previous results (Hõrak et al., 2003), where SRBC challenge caused only short-term elevation of markers of acute-phase response without any lasting effect on various plasma biochemicals, body mass or basal metabolic rate (BMR). However, costs arising from anti-SRBC response cannot be totally discounted because immune challenge with this antigen has been shown to elevate BMR in free-living great tits (*Parus major*) (Ots et al., 2001) and captive collared doves (*Streptopelia decaoto*) (Eraud et al., 2005). Moreover, production of antibody response against SRBC considerably reduced survival in incubating eiders (*Somateria mollissima*) (Hanssen et al., 2004).

We did not detect an effect of immune challenge on two different biomarkers of total antioxidant protection (TAS and AOP). This result contradicts our expectations based on the assumption that possible immunopathological damages, accompanying immune response, result from excess production of reactive species (e.g. Knight, 2000). For instance, inflammatory response accompanying strenuous exercise can reduce TAS (e.g. Ficicilar et al., 2003), indicating that in some situations, reduced TAS levels reflect hosts' inability to deal effectively with increased free radical load. However, an increase in TAS following exercise-induced oxidative stress has also been demonstrated (e.g. Vider et al., 2001), suggesting that, in some situations, increased TAS levels reflect compensatory enhancement of antioxidant defences. However, none of these changes could be associated with immune system activation in our study, which suggests that alterations of the total plasma antioxidant potential do not play any important role in forming the costs of SRBC-induced immune activation. Similarly, Alonso-Alvarez et al. did not find an effect of LPS injection on whole-blood antioxidant protection in zebra finches (Alonso-Alvarez et al., 2004).

Effects of carotenoid supplementation

Despite the pronounced effect of lutein supplementation on plasma carotenoid levels, we did not find any indication that this supplementation had affected the indices of immunocompetence measured in our study. Birds with almost depleted levels of carotenoids were capable of mounting similar primary and secondary anti-SRBC antibody titres and swelling responses to PHA as those circulating relatively high physiological doses of carotenoids. In this respect, our findings diverge from those of mammal studies (Jyonouchi et al., 1994; Kim et al., 2000a; Kim et al., 2000b), where lutein

supplementation has been shown to enhance immunoglobulin (IgG) production and/or T-cell proliferation. In chickens, lutein supplementation affected PHA-stimulated lymphocyte proliferation (Selvaraj et al., 2006), whereas no effect of antibody production against KLH (Selvaraj et al., 2006) or Newcastle disease virus (Haq et al., 1996) was detected. In moorhen (*Gallinula chloropus*) chicks, canthaxanthin supplementation enhanced PHA-response (Fenoglio et al., 2002). In passerines, two studies on captive zebra finches have found that lutein supplementation enhances PHA response (Blount et al., 2003; McGraw and Ardia, 2003); the latter study also found that carotenoid-supplemented birds mounted stronger antibody titres against SRBC. However, a range of dietary xanthophyll concentrations administered to male American goldfinches had no effect on several aspects of immunity and disease resistance (Navara and Hill, 2003).

One possible explanation for the discrepancy between these results might relate to the use of carotenoids for signalling purposes. Unlike greenfinches and goldfinches, whose carotenoid-based plumage coloration only signals their condition during moult, zebra finches can use dietary carotenoids to signal their current health status by flexibly changing their beak colour (a sexually selected signal) in response to circulating carotenoid levels (Blount et al., 2003). One might thus speculate that immunostimulatory effects of carotenoids are more likely to emerge in species possessing flexible (e.g. beaks and bare parts) rather than relatively static (e.g. plumage) carotenoid-based ornaments.

Independent of immune system activation, lutein supplementation affected fat deposition patterns as indicated by the significant increase in plasma triglyceride levels among supplemented birds (Fig. 2C). To our knowledge, such a phenomenon has not been previously described in an abundant carotenoid literature. We can exclude the possibility that carotenoid supplementation might have alleviated the coccidian-induced intestinal damage, known to suppress plasma triglyceride levels (Hõrak et al., 2004), because none of our treatments interfered with the dynamics of infection. Carotenoids are transported in blood by lipoproteins (mainly VLDL) (e.g. McGraw and Parker, 2006; McGraw et al., 2005b), which constitute the major part of plasma triglycerides. Thus, it seems that dietary lutein supplementation eventually leads to increased VLDL assembly in the liver, which inevitably results in elevation of circulating triglycerides as a by-product.

Antioxidant protection and carotenoids

Under the hypothesis that carotenoids significantly contribute to antioxidative protection, we predicted that individual plasma carotenoid levels correlate positively with measures of total antioxidativity. However, no such correlations emerged. We are confident that this lack of correlations cannot be ascribed to measurement techniques, because our estimates of total antioxidativity, obtained by two different assays, were highly correlated (Fig. 3). In line with our results, serum carotenoid concentration did not correlate with measures of antioxidant protection and serum concentration of reactive oxygen metabolites in a recent study of kestrel (*Falco tinnunculus*) nestlings (Costantini et al., 2006). Similarly, lutein supplementation to adult captive zebra finches had no direct effect on resistance of erythrocytes to oxidative lysis (Alonso-Alvarez et al., 2004). One possible explanation for these results would be that local actions of carotenoids in specific tissues are not reflected at the systemic level, so that plasma total antioxidant capacity is not affected. Such an explanation would be consistent with the results of Woodall et al., who demonstrated that despite the significant effect of zeaxanthin supplementation on plasma carotenoid levels in chicken, plasma lipid peroxidation was not affected by the treatments (Woodall et al., 1996). However, the lipid peroxidation in the liver was reduced by 78% when compared with the unsupplemented controls. Lack of correlation between plasma carotenoids and indices of total antioxidativity can also be reconciled with the results of an extensive meta-analysis of clinical studies of oxidative stress (Dotan et al., 2004), revealing that only under severe pathological conditions do all the indices of oxidative stress correlate with each other. However, at present, we cannot also totally exclude the alternative explanation, namely that systemic antioxidant properties of carotenoids in birds (except well-established protective effects on embryos and hatchlings) might not appear as important as previously thought, at least in situations where redox homeostasis is not threatened (see also Hartley and Kennedy, 2004).

In conclusion, our study found some evidence regarding the costs of humoral immune challenge and that some of these (reduced mass gain) can be alleviated by carotenoid supplementation. However, we did not find that immune challenge had induced any pathological damages that could be ascribed to oxidative stress. Carotenoid supplementation inclined birds to fattening, indicating that lutein interfered with lipid metabolism. Thus, although our results support the hypotheses of biological importance of carotenoids, they also exemplify the overwhelming complexity of their integrated ecophysiological functions.

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